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FOREWORD

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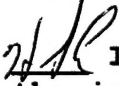
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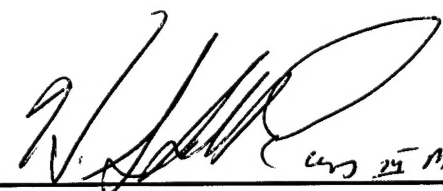
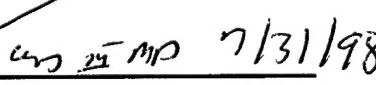
 
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Report for DAMD 17-96-1-6015
Abstract

During the second year of our HER4 grant we have made progress in developing methods and cell lines to study the unique role of HER4 in stopping breast cancer growth. We have made a full length HER4 cDNA and have used site directed mutagenesis to create dominant negative mutations both in the HER4 tyrosine kinase domain and as a truncation mutant that will block HER4 activation. We have created inducible promoter constructs that will express HER4 and dominant negative HER4 to more precisely control expression in breast cancer cell models. We have created two clonal lines expressing EGF receptor. HER4 chimera EHC in indicator cells. These lines express significantly different amounts of chimera. The biologic and, tyrosine kinase activity and growth suppression differs markedly in these two lines. We have created constructs to make transgenic mice to prove that HER4 will block breast cancer development. Lastly, we have successfully raised one antisera and are making the HER4 extracellular domain in baculovirus for use as immunogen for monoclonal antibodies.

Introduction

The human EGF receptor 2 gene (HER2) is amplified and overexpressed in 20-30% of invasive breast cancers (1). Moreover, numerous articles indicate that poor prognosis breast cancers exhibit increases in EGF receptor content and /or an EGF receptor autocrine loop with the production of $TGF\alpha$ (2, 3). Both EGF receptor and HER2 can interact with each other or with HER3 leading to growth and proliferation (4,5). However, the 4th member of the family, HER4, in preliminary studies appears to have a different output, differentiation rather than proliferation (6,7). Thus, the HER4 signal may slow the growth of breast cancer. Our tasks are to obtain definitive evidence that HER4 provides a different biologic signal to breast epithelium, i.e. differentiation rather than proliferation, and to elucidate the pathway, or elements of the pathway, that differ between HER4 and the original 3 members of this receptor family (EGF receptor, HER2 and HER3). To this end, we are creating molecular reagents and cell lines and devising new technology which will allow us to prove that HER4 sends a differentiation signal. These reagents should also give us the wherewithal to isolate the unique members of the HER4 pathway, substrates phosphorylated by this tyrosine kinase.

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Summary of Progress

Progress this year has been made in several different areas. First, we have now created a full length HER4 cDNA in an expression vector. Since all other HER4 cDNAs were covered by commercial licenses, we thought it was necessary to make our own clone so as to be unencumbered by industrial materials transfer agreements. We have used that clone to construct several types of dominant negative HER4s including a tyrosine kinase deficient full length and chimeric HER4 and a truncated HER4 that will be expressed at the cell surface and act as a dominant negative for HER4 activation. In addition, we have placed all of the important clones into the tetracycline-dependent inducible expression vector system. This will allow us to create stable lines in which we can turn off and on the expression of HER4 constructs. This is important since HER4 is growth inhibitory, and expression would make it difficult to create stable cell lines because growth inhibition will be selected against. Next, we have cloned several 32D cell lines expressing high or low amounts of the HER4 chimera. We have shown by FACS analysis and immuno-precipitation that they express different HER4 chimera levels. The overexpressing line has a constitutively active HER4 chimera and is very slow growing as befits a differentiation signal. We have also made progress constructing clones to make transgenic mice expressing the HER4 chimera and a baculovirus extracellular domain for the HER4 protein for use in the antibody [production. Our yeast 2 hybrid HER4 vector which we had hoped to use to isolate HER4 substrates proved to be inactive in yeast and we have made a new construct which we are testing at this time in another yeast strain.

Body

In year two we have concentrated on several tasks from our statement of work. Progress on task one, to devise breast cancer models distinguishing the growth promoting actions of the EGF receptor versus the differentiation promoting effects of HER4 is well under way. Task three is to create mechanisms for studying downstream HER4 P-Tyr substrates and task four is to extend these.

A: The creation of molecular reagents to study the growth promoting and differentiation effects of EGF receptor family members.

Full length HER4 cDNA clones, to our knowledge, are only available by materials transfer agreements with one of several companies. These type of agreements inhibit the free flow of information and we thought it important to make our own reagents. To this end in year one we cloned the cytoplasmic domain of the HER4 receptor and created a EGF receptor HER4 chimera. This year we decided for a number of reasons that we needed the entire HER4 molecule and therefore we isolated by PCR the HER4 extracellular domain again using RNA

isolated from the MDA-MB 453 cells. After high fidelity PCR and exhaustive sequencing, we selected a clone that was wild type with respect to amino acid sequence and created a full length HER4 cDNA expressed both in pcDNA and pLXSN. Figure 1 schematically outlines this clone and shows our strategy which we have now accomplished to create a kinase-dead full length HER4 and a kinase dead EGF receptor HER4 chimera. We decided these constructs were needed because we had shown that the chimera was capable of slowing growth both in the breast cancer cell line BT 474 and in 32D cells. The appropriate control to show that this was due to the HER4 tyrosine kinase is a kinase-dead construct. These have now been made and will be transfected in the cells.

Another type of dominant negative construct is shown in Figure 2. If one can express the extracellular domain anchored by a transmembrane domain of EGF receptor family members, it is possible to block ligand dependent activation. We've used the full length HER4 cDNA and placed a stop codon right after the tri-basic amino acid anchoring sequence in the juxtamembrane area of the molecule. High level expression of this should allow us to stop the differentiation effect of Heregulin in MDA-MB 453 cells and other cells in which Heregulin causes differentiation. This would allow us to prove that the HER4 signal is a differentiation one.

Lastly in the area of creation of new reagents we realize that since the HER4 signal is growth inhibitory it may be difficult to create cell lines expressing this molecule. Therefore we have cloned the full length HER4, the chimera, as well as the kinase dead version of these two molecules and the HER4 dominant negative cytoplasmic domain into the tetracycline off system. This is felt to be one of the best inducible promoter systems. We will shortly transfect this two plasmid system into test cell lines to determine whether we can get high level, inducible expression by removing tetracycline. If we can, this will greatly speed our progress.

B. The Action of the EGF Receptor HER4 Chimera and 32D Cells

Last year we reported that populations of chimeric transfected 32D cells underwent growth slowing in response to ligand. This was in line with our hypothesis that HER4 sends a differentiation and not a proliferative signal. This year we have created two clones which express different levels of the HER4 chimera. EHC-2 expresses a high level and EHC 11 expresses 10-20% as much HER4 chimera. Figure 4 shows a FACS analysis of HER4 chimera expression. Figure 5 shows the amount of tyrosine phosphorylated HER4 chimera in the two clones. The high expressor, EHC 2, exhibits constitutive auto phosphorylation of HER4. The addition of EGF dramatically increases tyrosine phosphorylation but the most important aspect is that there is phosphorylation in a non-ligand state. This correlates with the growth curve Figure 6, which demonstrates that EHC2 grows much more slower and that EGF blocks IL-3

dependent growth in this clone. The lower expressor, EHC clone 11, contains HER4 chimera and it can be activated in a EGF dependent manner. However there is no constitutive phosphorylation and this clone (as shown in Figure 6) grows much faster. We will be working with these two clones to tease apart the response curves and hopefully the pathways involved in growth suppression.

C. Production of Antisera

We have not been able to purchase commercial antisera with high affinity to HER4 and therefore we continue to try to make antibodies. The original GST immunizations did not produce antisera with significant titer. We have recently finished one series of immunizations with 100 amino acid fragment of HER4 and have now isolated an immunoprecipitating antibody. Figure 7 shows that HER4 antibody will immunoprecipitate tyrosine phosphorylated HER4 from clone 2. The level of detection in clone 11 is much less and this demonstrates that while we have antisera, its probably not of sufficient titer to recognize cells expressing normal amounts of HER4. We have recently created another GST HER4 epitope and have immunized a series of rabbits which will hopefully give us higher titer antisera.

In addition, we have taken the extra cellular domain of HER4 and placed it into the p-FASBAC vector and are creating a HER4 extracellular domain baculovirus. We will use this for immunogen. This soluble protein will have the 6 histidine tag at the end and therefore we expect to be able to purify the extracellular domain quite nicely using nickel columns. Once available, this immunogen will be used for both polyclonal and monoclonal antibody production. Our aim is to create a monoclonal antibody capable of recognizing the human HER4 in paraffin embedded sections. In this manner we will be able to do translational research with archived tissues allowing us to determine whether the HER4 expression level is an independent prognostic variable for breast cancer survival or whether it is a modifier of HER2 predicted prognosis. Our hypothesis would be that high levels of HER4 expression would attenuate the poor prognosis seen with increased levels of HER2 expression.

D. Development of the Yeast 2 Hybrid System

The Gal 4:HER4 construct described in January's progress report was active in mammalian cells when expressed. Unfortunately the yeast strain used for the Gal 4 system did not result in tyrosine phosphorylation of this construct. We have recloned this construct into the LEX A system and are testing to see if it will be tyrosine phosphorylated in this other yeast strain. This will enable us to pursue yeast 2 hybrid experiment, to isolate HER4 substrates.

E. Creation of HER4 chimera expressing transgenic mice

As indicated in January's progress report we've decided one of the most direct ways to determine whether the HER4 chimera could prevent breast cancer will be to use animal models. Our colleague David Lee has created a number of TGF α transgenic mice with TGF α targeted to the mammary gland. These targeted mice develop breast cancer at an accelerated rate. We have now cloned the EGF receptor HER4 chimera into the two vectors used to target mammary tissue. The first is WAP (Whey Acidic Protein) which is expressed particularly in pregnancy and during lactation. The second is the MMTV promoter which is expressed beginning early in puberty and can be induced to be expressed earlier by steroid hormone injections in the mice. Dr. Lee has created TGF α mice with both these promoters and breast cancer results at different times during mouse development. We have already made our first injection into pronuclei with the WAP promoter construct and will shortly be injecting the MMTV. Since breast development is triggered by EGF receptor family members presumably activating the EGF receptor, the expression of the chimera may have a phenotype, developmental anomalies of the mouse breast after the activation of the EGF receptor HER4 chimera by endogenous ligands for the EGF receptor such as TGF α or amphiregulin. Once we determine whether there is any phenotype in the transgenic mice, these will be bred to Dr. Lee's TGF α producing mice. We will determine if we can reduce the incidence of mammary carcinogenesis with EGF receptor HER4 chimera.

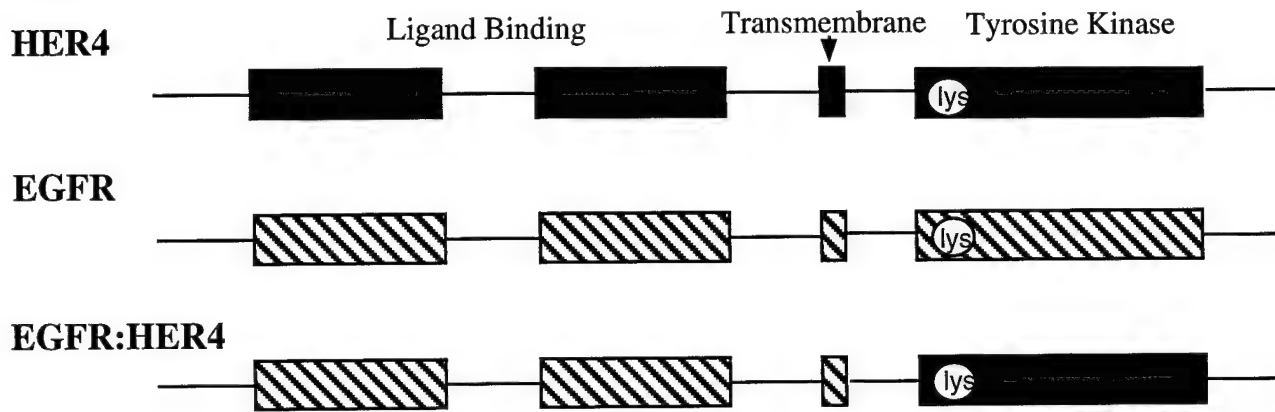
Future Directions

We have now created a number of molecular reagents that will allow us to look at breast cancer cell lines, indicator cells and transgenic mice to formally test our hypothesis that HER4 causes differentiation of breast cells. While this has been suspected by a number of researchers, the experiments planned with the HER4 chimera, the HER4 kinase dead chimera and the transmembrane dominant negative receptors should allow us to test in several cell lines this hypothesis. In addition, we will be able to use our inducible promoters and our transgenic mice to show that activated HER4 can block breast carcinogenesis. The animal model is particularly exciting in this regard. These experiments will allow us to choose the best model in which to look for downstream elements in the HER4 pathway.

Conclusions

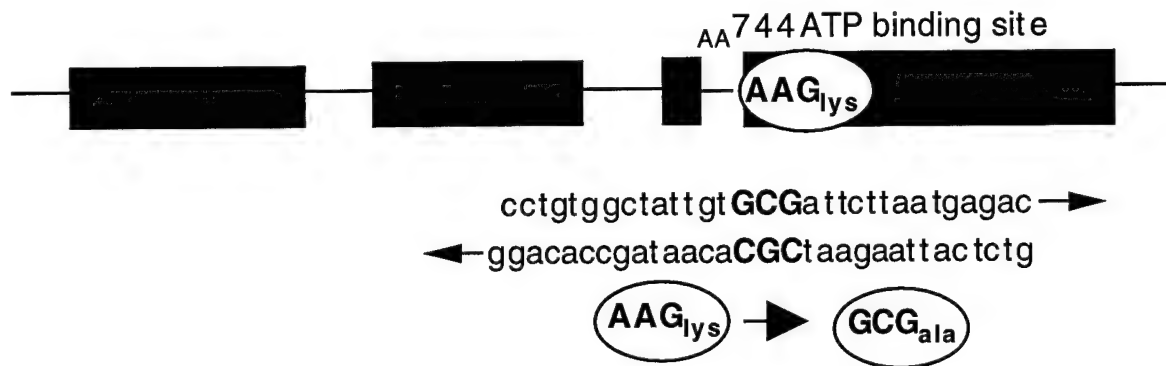
In summary, during the first two years of this proposal, we have created the wherewithal to study the differentiation signals sent by HER4. The creation of dominant negative mutants and transgenic mice in which we have reason to believe the HER4 chimera will block tumorigenesis will allow us to determine whether there is a new avenue of anti-carcinogenesis, the HER4 receptor. In addition, a number of strategies will begin in year 3 to identify the downstream signaling components. If as we suspect, HER4 is antiproliferative and causes differentiation as well as blocking tumorigenesis, then understanding the HER4 pathway and devising small molecules to activate it could produce novel therapies for breast cancer. Conversely, loss of constituent members of the HER4 pathway may act like loss of a tumor suppressor gene.

Structure of HER4, EGFR, EGFR:HER4 chimera



HER4 and EGFR:HER4 kinase dead

HER4 kinase dead



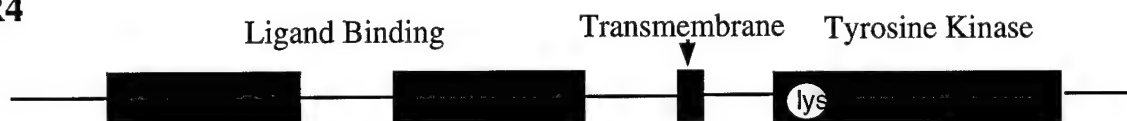
EGFR:HER4 kinase dead



Figure 1. Expression vectors for use in HER4 experiments. This year we isolated the HER4 extracellular domain and constructed a full length HER4 cDNA. In addition, we created two kinase dead mutants by site-directed mutagenesis.

HER4 dominant negative

HER4



HER4 dominant negative

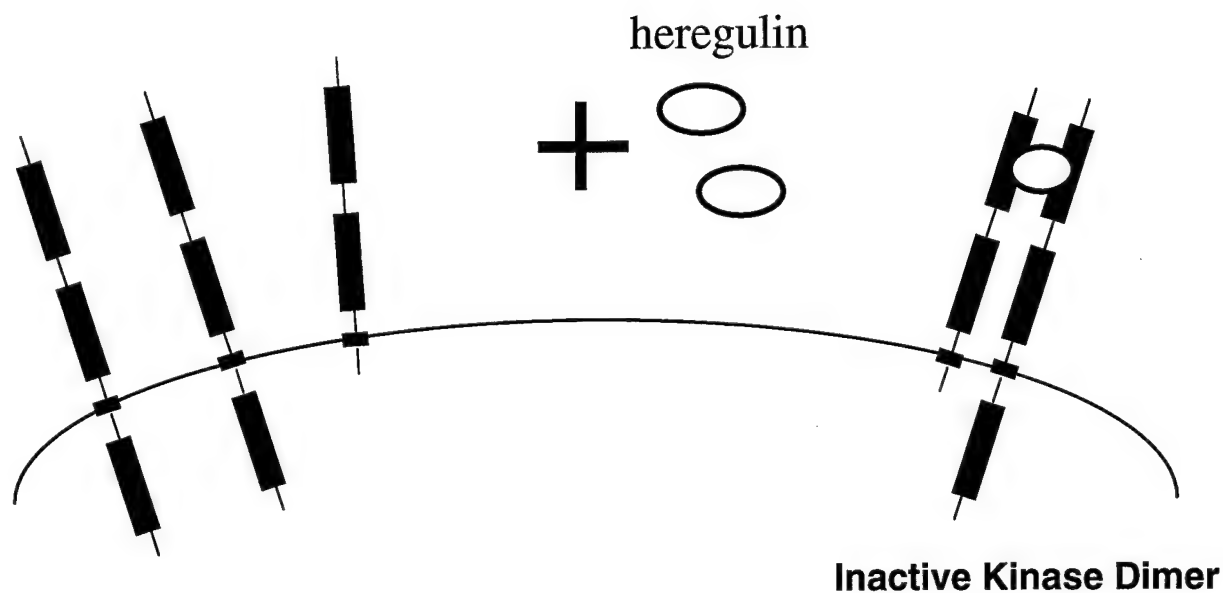


Figure 2. Construction of a HER4 dominant negative expression vector. The full length HER4 was used. A stop codon was placed at amino acid 684 just beyond the tribasic amino acid juxtamembrane region that anchors the HER4 molecule in the membrane. When expressed, this construct is predicted to prevent activation of HER4.

Tetracycline dependent inducible expression of HER4, and EGFR:HER4 clones

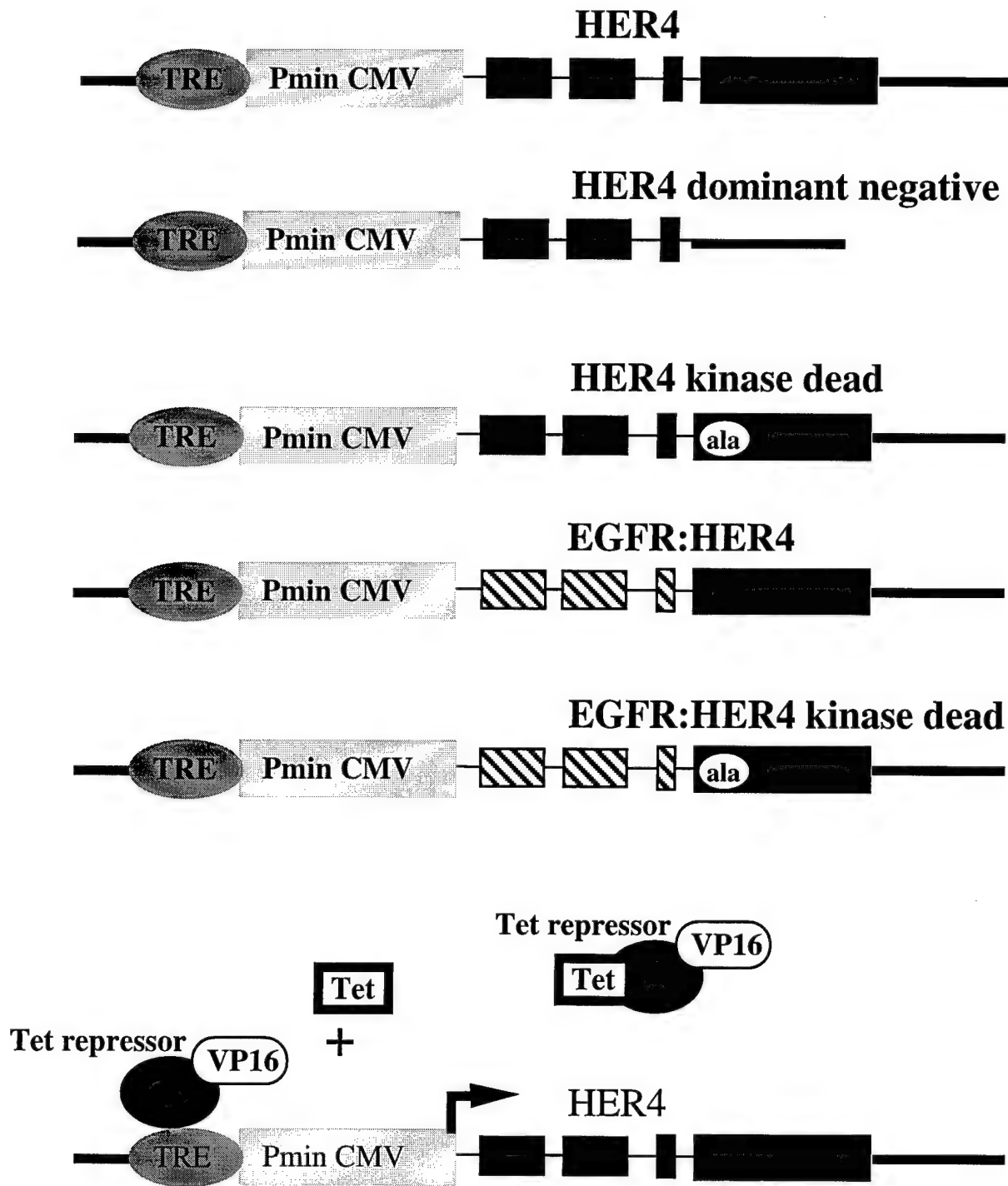
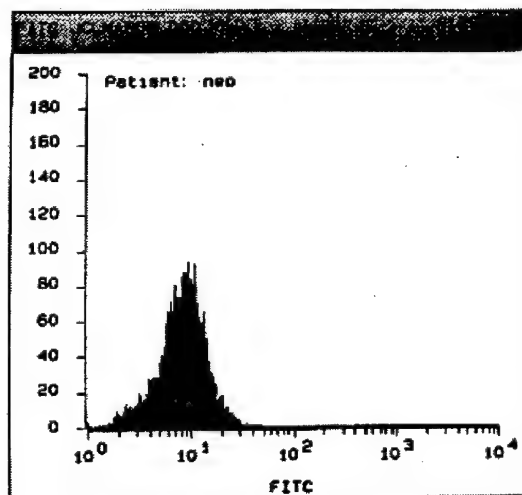


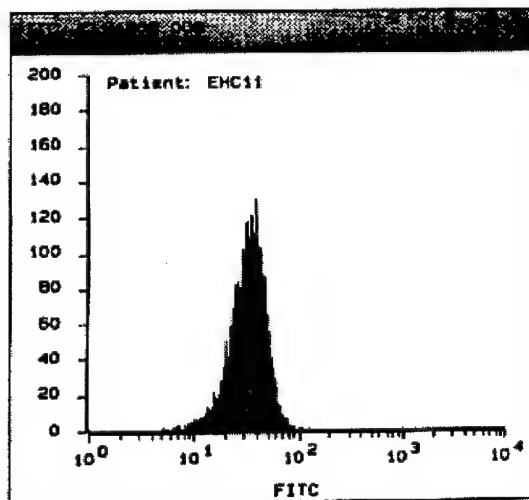
Figure 3. Inducible promoter for HER4 constructs. Each of the important HER4 cDNAs were placed in the "Tet off" vector which will allow the creation of stably transfected lines with tightly-controlled HER4 construct expression.

Fac Scan analysis of EGFR expression on EGFR:HER4 clones

vector control



EGFR:HER4
clone 11



EGFR:HER4
clone 2

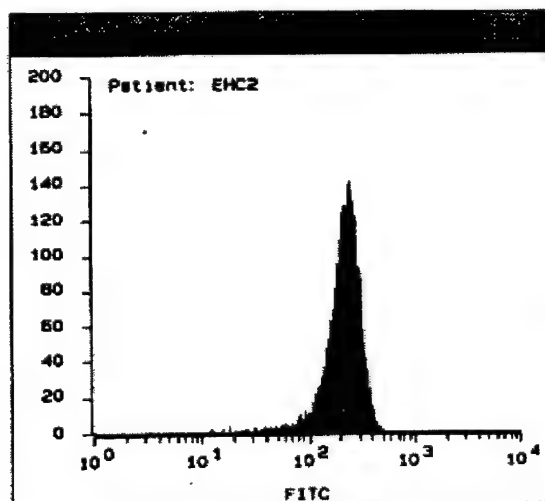


Figure 4. Analysis shows that we have created two 32D cell clones with constitutively high (EHC2) and low (EHC11) levels of expression. 32D cells were washed and incubated with anti-EGFR antibody, followed by an incubation with anti-rabbit FITC conjugated secondary antibody. The cells were washed once more and analysed by flow cytometry for FITC staining.

EGF Stimulation of EGFR:HER4 32D clones

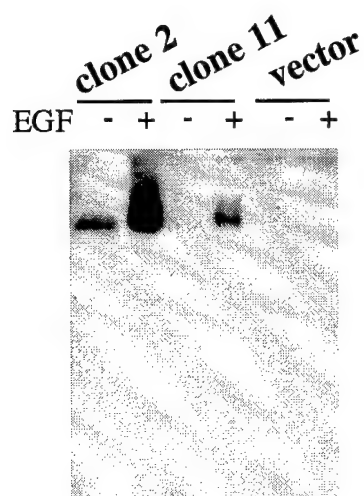


Figure 5. The high (EHC2) and low (EHC11) expressing clones exhibit different levels of ligand-independent (-EGF) and ligand-dependent (+EGF) tyrosine phosphorylation. 32D cells were removed from IL3 containing medium for 3 hours. The cells were then stimulated with 0.1 μ g/ml EGF for 90 seconds, washed, lysed and immunoprecipitated with anti-EGFR antibody. The immunoprecipitate was then run on a 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. Clone 2 expresses the EGFR:HER4 chimera at 5-10 times the level of clone 11. The chimera is constitutively activated, but can be further stimulated by EGF. Whereas clone 11 expresses the chimera to a lower level and is active only with the addition of EGF.

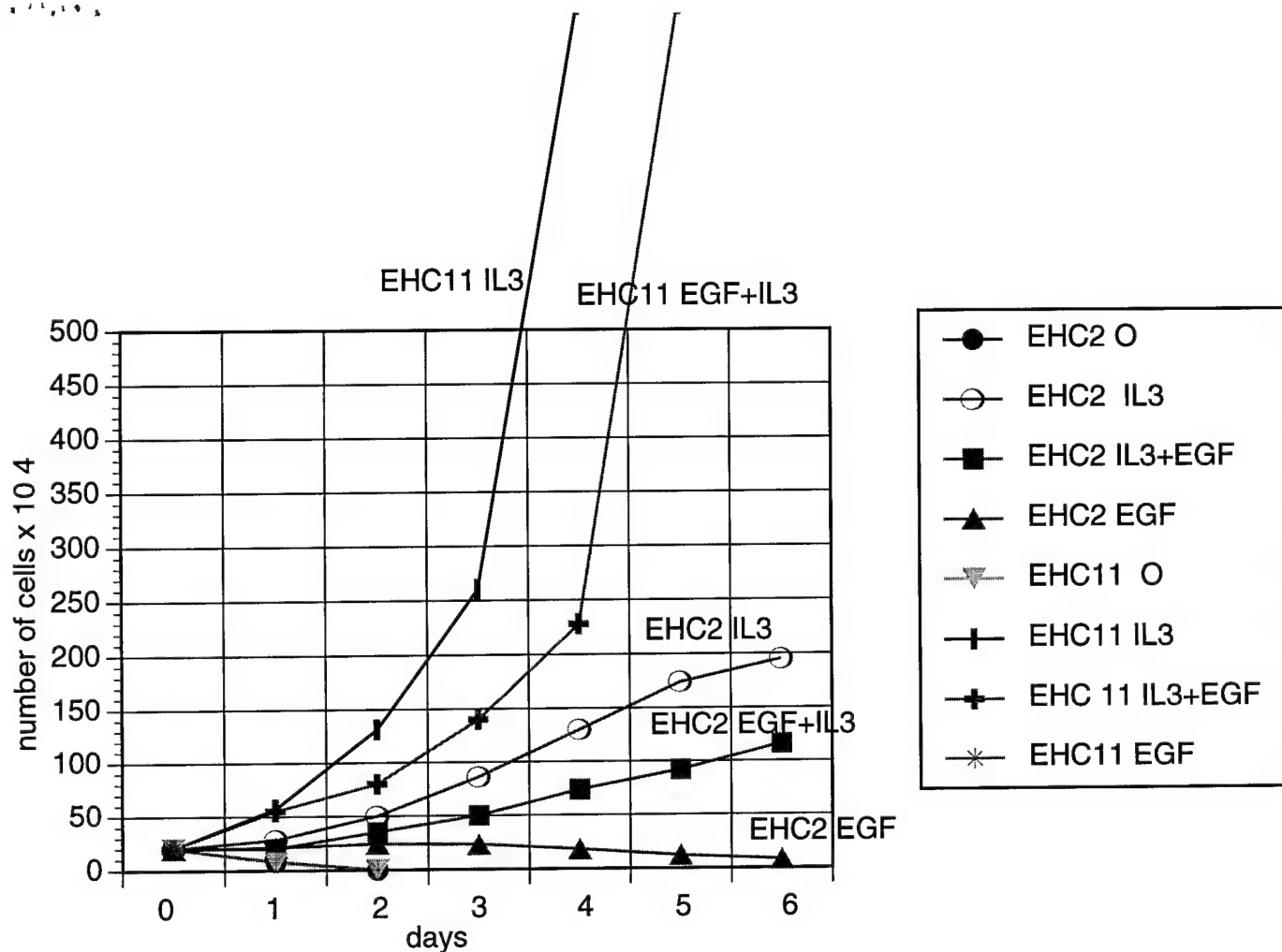


Figure 6. Growth curve of EHC-expressing 32D clones. High expressing EHC2 clone shows a different growth pattern compared to low expressing EHC11 clone. EHC11 cells grow exponentially with the addition of IL3, and slightly slower with both IL3 and EGF. With no addition or addition of EGF alone the EHC11 cells die quickly; all are dead within two days. Thus the level of HER4 chimera expression in EHC11 is insufficient for biological signalling. In contrast, EHC2 which expresses EHC at high levels and exhibits an autoactivated HER4 kinase, grows slowly in the presence of IL3, and even slower with the addition of both IL3 and EGF. However, addition of EGF prevents cell death, and EHC2 cells remain viable many days after EHC11 cells have died.

Immunoprecipitation of EGFR:HER4 from 32D clones using HER4 antibody

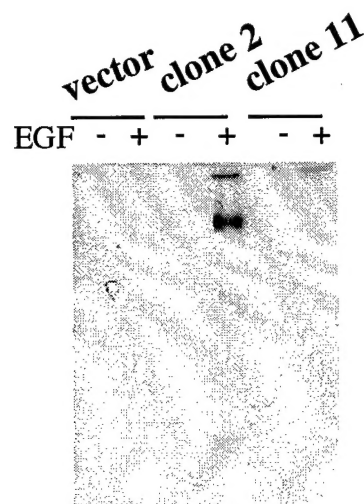
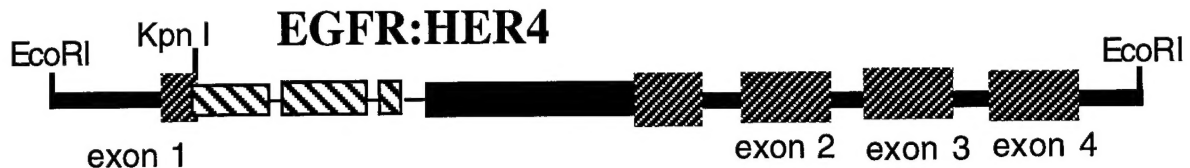


Figure 7. Anti-HER4. A GST-HER4 fusion protein was used to raise anti-HER4 antibody. 32D cells were removed from IL3 containing medium for 3 hours. The cells were then stimulated with 0.1 μ g/ml EGF for 90 seconds, washed, lysed and immunoprecipitated with anti-HER4 antibody. The immunoprecipitate was then run on a 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. In addition, when the blot is overexposed a faint band is seen with clone 11. Thus we have a specific anti-HER4 antibody but the affinity/titer is less than that of 1382, the EGFR antibody used in Figure 5.

EGFR:HER4 chimera cloned into WAP and MMTV vectors: for use in creating transgenic mice

WAP: expression begins with pregnancy



MMTV: expression begins with first menstrual cycle

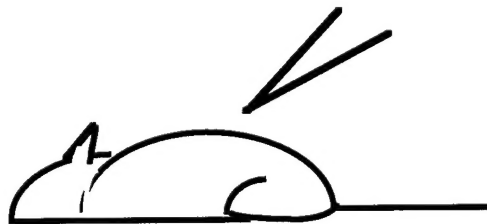


Figure 8. In collaboration with David Lee we are making EHC bearing transgenic mice. These transcripts will be expressed during puberty (MMTV) or pregnancy (Whey Acidic Protein, WAP). The HER4 kinase domain will become activated when EGF receptor ligands are expressed either in development of normal mammary gland or during TGF α -induced mammary tumorigenesis induced in the TGF α transgenic mice.

HER4 baculovirus expression

HER4 extracellular domain cloned into pFastBac baculovirus expression plasmid

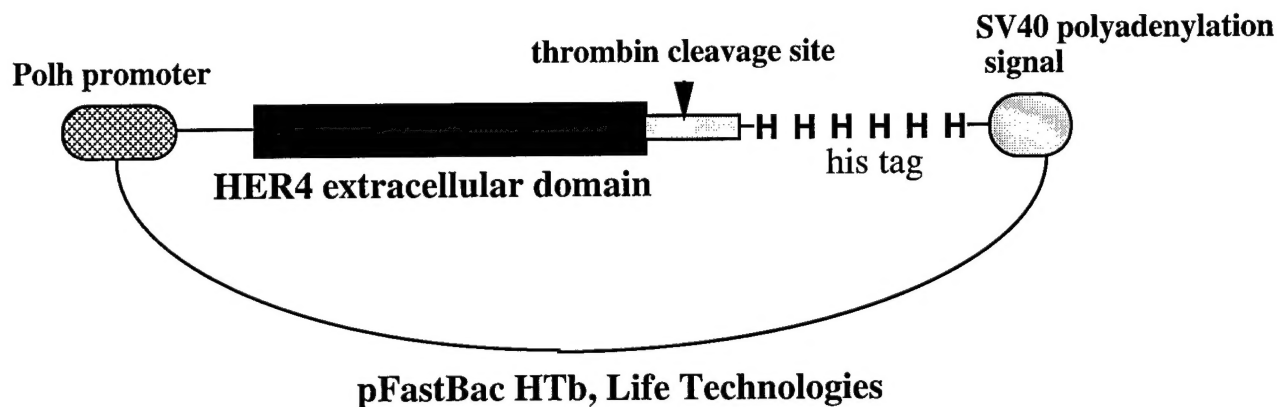


Figure 9. Creation of an extracellular domain HER4 baculovirus. Purification with Ni^{++} columns that bind to the 6-His tag will provide immunogen for monoclonal antibody formation.

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